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7 **Monitoring protein hydrolysis by pepsin using pH-stat: *In vitro* gastric digestions**  
8 **in static and dynamic pH conditions**

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10

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19 **Abstract**

20           This study intends to demonstrate that acid titration at low pH is very well adapted to the  
21 monitoring of pepsin activity. After a description of the underlying principles, this approach was used  
22 during *in vitro* gastric digestions of a model of complex food containing 15 wt% of whey proteins,  
23 according to both static (2h at pH = 3, Infogest protocol) and dynamic pH conditions (from pH 6.3 down  
24 to 2 in 1h). Pepsin activity was quantitatively assessed in all experiments through the calculation of  
25 degrees of hydrolysis (DH). Final values of 3.7 and 3.0 % were obtained in static and dynamic pH  
26 conditions, respectively, and validated using an independent method. Results also show that about 92%  
27 of the peptides were detected at pH = 3, and 100% for  $\text{pH} \leq 2.5$ . Overall, the proposed approach proved  
28 to be very worthy to study protein hydrolysis during *in vitro* gastric digestions.

29

30 **Keywords**

31           pH-stat; Titrimetry; Pepsin; Aspartic proteases; Proteolysis; Hydrolysis; Whey proteins; *in vitro*  
32 digestion

## 33 1 Introduction

34 Stomach is the first compartment of the digestive tract after food has been swallowed. During  
35 the gastric phase of digestion, the food is mixed with fluids that dramatically lower its pH to about 2 in  
36 1 to 2 h thanks to the production of HCl by parietal cells (Dressman, 1986; Malagelada, Go, &  
37 Summerskill, 1979). Enzymes are also secreted, notably pepsin and gastric lipase, initiating the  
38 hydrolysis of proteins and lipids, respectively. Compared to the intestinal phase, the extent of hydrolysis  
39 is relatively limited at the gastric stage, up to typically 10-15% (Griffiths, 2012; Norton, Wallis,  
40 Spyropoulos, Lillford, & Norton, 2014). However, these enzymatic reactions are of particular interest  
41 to understand the effects of food properties on the gastric emptying kinetics, which largely govern the  
42 kinetics of appearance of nutrients in the peripheral blood, as shown for amino acids (Le Feunteun et  
43 al., 2014) and glucose (Marathe, Rayner, Jones, & Horowitz, 2013).

44 In the field of food sciences, *in vitro* digestion experiments constitute a powerful means to  
45 investigate the effects of food composition and structure on digestion, which is triggered by the growing  
46 interest of designing foods with particular nutritive properties (Golding & Wooster, 2010; Guerra et al.,  
47 2012; Ménard et al., 2014; Wickham, Faulks, & Mills, 2009). They present the great advantage of being  
48 a relatively fast and inexpensive way to study food digestion, and allow to avoid, or at least to back up,  
49 *in vivo* studies, which are accompanied by high cost, complexity and variability (inter and intra  
50 individual). *In vitro* digestion protocols mostly fall into two categories: dynamic and static approaches  
51 (Guerra et al., 2012). On the one hand, dynamic protocols try to reproduce the main events encountered  
52 within the gastro-intestinal tract using dynamically-controlled transit fluxes, pH, mechanical constraints,  
53 and/or digestive fluid secretions. On the other hand, mono-compartmental static models offer simplicity,  
54 which explains a more widespread use. They generally consist in subsequently immersing the food in  
55 simulated gastric and intestinal fluids to perform gastro-intestinal digestions. Moreover, to overcome  
56 the lack of homogeneity in the protocols, a harmonized static protocol was recently proposed (Minekus  
57 et al., 2014), based on a consensus of international experts involved in the Infogest COST action  
58 (<http://www.cost-infogest.eu>).

59 pH-stat titration is probably the most commonly employed method to monitor hydrolysis

60 reactions, and therefore appears as a highly complementary approach to *in vitro* digestion experiments.  
61 In adequate pH conditions, lipid and protein hydrolyses may lead to the release or the consumption of  
62 protons. In response, an automatic addition of an acidic or basic solution maintains the pH constant, so  
63 that the titration rate is proportional to the reaction rate. The quantity of titrant added can also be  
64 converted into a degree of hydrolysis of the considered nutrient. Overall, pH-stat is thus a simple, fast,  
65 reproducible, and nondestructive way to investigate the kinetics of enzymatic digestion of foods. In this  
66 field of research, this method is very popular to monitor lipolysis reactions during a simulated intestinal  
67 digestion at pH 7 or above, during which a base is used to titrate the protons released by the fatty acids  
68 produced (McClements & Li, 2010; Williams et al., 2012; Wooster et al., 2014). In such pH conditions,  
69 it is also possible to detect protein hydrolysis (Asselin, Hébert, & Amiot, 1989; Camacho, González-  
70 Tello, Páez-Dueñas, Guadix, & Guadix, 2001; Spellman, McEvoy, O’Cuinn, & FitzGerald, 2003;  
71 Turgeon, Bard, & Gauthier, 1991), hence enabling intestinal proteolysis kinetics to be characterized as  
72 well.

73 In a previous study (Mat, Le Feunteun, Michon, & Souchon, 2016), we showed that pH-stat  
74 titration can be used during the intestinal phase of *in vitro* digestion to continuously monitor both the  
75 lipolysis and proteolysis kinetics of a model of complex foods. The approach proved efficient at  
76 exploring the influence of some food properties on the enzymatic reactions, which may be of use for a  
77 rapid screening of food compositional and/or structural effects on digestion. The same principles are  
78 never used during the course of gastric digestion experiments, even though it seems theoretically  
79 possible to detect protein hydrolysis by pH-stat in acidic conditions (Nielsen, 1997). Associating pH-  
80 stat titration during the gastric phase of *in vitro* digestion might indeed prove useful in the ongoing effort  
81 to gain knowledge on the effects of food properties on the gastric phase of digestion. When considering  
82 the great number of articles that investigate the effects of food structure on gastric digestion, and the  
83 popularity of the pH-stat technique for the intestinal phase, it is even surprising that the possibility of  
84 resorting to pH-stat to assess pepsin activity received little attention so far.

85 The aim of this study is to demonstrate that pepsin proteolysis can be easily and accurately  
86 monitored by pH-stat during the course of *in vitro* gastric digestions of an emulsion-type food model

87 made of 15 wt% of whey proteins and 10 wt% of oil, which constitutes a good model of complex food  
88 matrices. Whey proteins are widely used in the food industry and are known to be quite resistant to  
89 pepsin action in their native forms, in particular  $\beta$ -lactoglobulin, the main protein of bovine whey  
90 (Asselin et al., 1989; Kim et al., 2007). This protein system may therefore be considered as a model of  
91 foods that are only slightly hydrolyzed in the stomach, hence suitable to test the sensitivity of the  
92 proposed approach. Two different protocols of *in vitro* gastric digestions were investigated. The first  
93 corresponds to the gastric phase of the recently proposed harmonized static *in vitro* digestion protocol  
94 (Minekus et al., 2014), during which the pH was maintained constant at 3.0 for 2 h. The second protocol  
95 was designed to achieve controlled acidifying kinetics, from the native pH of the food to pH 2 in about  
96 1 h, before maintaining the medium at pH 2.0 during another 1 h. This latter constitutes a proposal to  
97 go beyond purely static digestions, and advance towards dynamic pH conditions of digestion, more in  
98 line with the physiologic reality (Dressman, 1986; Malagelada et al., 1979). For both types of  
99 experiment, it is shown how to convert titrant volumes into degrees of hydrolysis, with a validation of  
100 our results according to independent measurements using the ortho-phthalaldehyde (OPA) method.

101

## 102 2 Theoretical considerations

103 Fig. 1 shows the protein hydrolysis reaction and schematically illustrates the principles of the  
104 pH-stat titration method at both  $\text{pH} \geq 7$  and  $\text{pH} \leq 5$ . Although no experiment was performed at neutral  
105 or basic pH in the present study, it is worth reminding the principles of pH-stat titration in such  
106 conditions to draw a parallel with the principles of acid titration of proteolysis at acidic pH.

107

108 *pH-stat titration at  $\text{pH} \geq 7$* : As illustrated in Fig. 1, during the hydrolysis of a peptide bond, one  
109 carboxylic group ( $-\text{COOH}$ ) is produced along with one amino group ( $-\text{NH}_2$ ), at the C-terminus and N-  
110 terminus of the two peptides produced, respectively. At neutral or basic pH, the neo-formed carboxylic  
111 groups release their proton, which may therefore be titrated by a basic solution (NaOH typically).  
112 However, most of the coproduced amino groups also consume a proton to form ammonium groups at

113 neutral pH, hence counteracting the majority of protons released by the carboxylic groups. To convert  
 114 the volumes of basic titrant into degrees of hydrolysis (DH in %) of proteins, which represent the  
 115 percentages of hydrolyzed peptide bonds, one therefore needs to take the mean degree of dissociation  
 116 of amino groups at the pH considered,  $\alpha_{NH_2}$ , into account according to the following equation (Spellman  
 117 et al., 2003; Turgeon et al., 1991):

118 *Equation 1*

$$119 \quad DH = 100 \times \frac{V \times N}{m \times h_{tot}} \times \frac{1}{\alpha_{NH_2}}$$

120 where  $V$  is the volume of titrant (mL),  $N$  its normality (meq/mL),  $m$  is the protein mass (g), and  $h_{tot}$  is  
 121 the number of peptide bonds per gram of proteins. The mean degree of dissociation can be estimated  
 122 from the  $pK_A$  of the amino groups through (Adler-Nissen, Eriksen, & Olsen, 1983):

123 *Equation 2*

$$124 \quad \alpha_{NH_2} = \frac{10^{pH-pK_A(NH_2)}}{1 + 10^{pH-pK_A(NH_2)}}$$

125  
 126 *pH-stat titration at  $pH \lesssim 5$ :* In acidic conditions, the chemical equilibria are reversed. The  
 127 acid/base equilibrium of the amino groups is entirely in favor of the protonated form ( $-NH_3^+$ ). One  
 128 proton is thus consumed for each hydrolyzed peptide bond, and may therefore be titrated by an acid  
 129 solution, HCl typically. Meanwhile, the co-produced carboxylic groups are in equilibrium between the  
 130 protonated and deprotonated forms. The protons that are released from carboxylic groups therefore  
 131 partly counteract those consumed by amino groups. Thus, at acidic pH, one should take into account the  
 132 mean degree of association of the carboxyl groups ( $1 - \alpha_{COOH}$ ) to convert volumes of acid titrant into  
 133 DH:

134 *Equation 3*

$$135 \quad DH = 100 \times \frac{V \times N}{m \times h_{tot}} \times \frac{1}{1 - \alpha_{COOH}}$$

136 with  $\alpha_{COOH}$ , the mean degree of dissociation of the carboxylic groups produced, which can also be

137 estimated through:

138 *Equation 4*

$$139 \quad \alpha_{COOH} = \frac{10^{pH-pK_A(COOH)}}{1 + 10^{pH-pK_A(COOH)}}$$

140

141 *Acid titration during acidifying kinetics:* If Equation 3 can be used to estimate degrees of  
142 hydrolysis when pH is maintained constant by acid titration, the situation is slightly different during the  
143 course of an acidification because most of the acid added is used to counteract the buffering capacity of  
144 the sample. The easiest way to take this buffering effect into account is to run a blank experiment (with  
145 no enzyme), so that the enzyme activity is related to the difference between the volumes of acid added  
146 in presence and in absence of enzyme. With such an experimental strategy, Equation 3 becomes:

147 *Equation 5*

$$148 \quad DH = 100 \times \frac{(V_{DD}(pH) - V_{BD}(pH)) \times N}{m \times h_{tot}} \times \frac{1}{1 - \alpha_{COOH}(pH)}$$

149 with  $V_{DD}(pH)$  and  $V_{BD}(pH)$  the volumes of acid added (mL) as a function of pH during digestion (DD)  
150 and before digestion (BD), *i.e.* in presence and absence of the proteolytic enzyme, respectively. It is  
151 noteworthy that  $V_{DD}$ ,  $V_{BD}$  and  $\alpha_{COOH}$  all vary with pH, and that the subtraction between  $V_{DD}$  and  $V_{BD}$  is  
152 only valid for identical pH values, not time. To determine the evolution of DH with time, one therefore  
153 needs to interpolate  $V_{DD}$  and  $V_{BD}$  at identical pH values to solve Equation 5, before analyzing the results  
154 as a function of the corresponding times in the DD experiments. As a matter of course, one also needs  
155 to estimate the values taken by  $\alpha_{COOH}$  at the considered pH to solve Equation 3 and Equation 5. Such  
156 data will be presented as a part of the results and discussion section.

157

### 158 **3 Materials & Methods**

#### 159 **3.1 Materials**

160 Whey protein isolate (WPI) powder, branded as Prolacta 95 (95 wt% on dry powder, Nx6.38),

161 was purchased from Lactalis, France. Rapeseed oil (Fleur de colza, Lesieur, France) was bought at a  
162 local supermarket. Pepsin (P6887) and pepstatin (P5318), both of porcine origin, were obtained from  
163 Sigma-Aldrich, France. The pepsin enzymatic activity was determined according to the assay described  
164 in (Minekus et al., 2014). Water was Milli-Q water and all other materials were of standard analytical  
165 grade.

166

### 167 **3.2 Model food**

168 An oil-in-water liquid emulsion containing 15 wt% of whey proteins and 10 wt% of rapeseed  
169 oil was prepared. It was chosen for comparison purposes with our previous study (Mat et al., 2016), and  
170 because it can be considered as an interesting model of complex foods. It was prepared in a semi-  
171 industrial way as follows. WPI was dispersed in water with a powder liquid mixer (CMS 2000 module,  
172 IKA, Germany) connected to a 30 L vessel in a recirculating arrangement. Proteins were allowed  
173 rehydrating for 1 h at the minimal circulation speed to avoid heating. Then, oil was poured into the  
174 vessel and the rotation speed of the mixer was adjusted to 4,770 rpm to create enough shear to prepare  
175 a coarse emulsion. After a 15 min interval (to reach a steady state regime), the emulsion was  
176 progressively collected and further refined in a two-stage high pressure homogenizer (NS2002H, GEA  
177 Niro Soavi Laboratory, Italy), operating at 400 and 100 bar in the first and second stages, respectively.  
178 Collected fractions were then stored at -80 °C and freeze-dried (Lyofal, France). The dried emulsion  
179 was rehydrated on the day of the *in vitro* experiments to recover a final composition of 15 wt% of  
180 proteins and 10 wt% of oil, with stable oil droplet sizes centered at 3.8 µm.

181

### 182 **3.3 Gastric *in vitro* digestions**

183 Gastric *in vitro* digestion experiments were carried out in 50 mL Schott bottles immersed in a  
184 jacketed beaker filled with water that was maintained at 37 °C by heat water circulation in the double  
185 wall. The whole set was mounted on a magnetic stirrer operating at 375 rpm, and was equipped with an

186 automatic pH-stat titration unit (Titroline7000, VWR, France) using a data acquisition frequency of 1 s<sup>-1</sup>.  
187 <sup>1</sup>. The digestion protocol followed all the recommendations of (Minekus et al., 2014), except that  
188 NaHCO<sub>3</sub> salts were replaced by NaCl in the electrolyte solutions to avoid possible pH variations induced  
189 by the degassing of CO<sub>2</sub> (Mat et al., 2016). Briefly, based on a final digestion volume of 30 mL, 7.5 g  
190 of the model food were rehydrated and weighted in the bottle. 7.5 mL of simulated salivary fluid (SSF)  
191 (with no amylase) and 13.5 mL of simulated gastric fluid (SGF) (with no pepsin) were added and were  
192 let to equilibrate at 37°C. As further detailed, the *in vitro* gastric digestion experiments were launched  
193 for 2 h according to either static or dynamic pH conditions. All measurements were made in triplicates.  
194

### 195 **3.3.1 Static pH conditions (pH = 3):**

196 The pH of the reaction medium was first adjusted at 3.0 using a 1 N HCl solution, and the  
197 automatic titration unit was programmed in a pH-stat mode to maintain the pH constant using a 0.05 N  
198 HCl solution as a titrant. The gastric digestion experiment was started by adding 1.5 mL of a pepsin  
199 solution to achieve a 2000 U/mL activity in the mixture and an initial gastric volume of 30 mL. Titration  
200 was immediately turned on. At the end of the experiment, a 200 µL sample was collected for further  
201 analysis of the degree of hydrolysis using the ortho-phthalaldehyde (OPA) method.  
202

### 203 **3.3.2 Dynamic pH conditions (from pH 6.3 down to 2)**

204 This second protocol was designed to mimic the progressive acidification of the stomach content  
205 that takes place *in vivo*, and test whether pepsin activity could still be monitored by titrimetry with these  
206 more complex operating conditions. To this end, the initial pH of the reaction medium (pH 6.3) was not  
207 modified initially. The automatic titration unit was still programmed in a pH-stat mode but with a target  
208 pH of 2.0, a 1 N HCl titrant solution, and a flux of titrant of 0.031 mL/min down to pH 2. The HCl  
209 concentration and the flux of titrant were both set according to preliminary tests so as to recover a pH  
210 decrease from ~ 6.3 down to 2 in about 1h of experiment, with a neither too small nor too large volume  
211 of added HCl (*i.e.* < 3 mL, corresponding to less than 10% of the initial volume).

212 Three different sets of experiments were carried out with these acidifying conditions. In the first  
213 set (BD, referring to “Before Digestion”), 1.5 mL of water (no pepsin) were added before launching the  
214 titration experiment in order to determine the buffering capacity of the undigested test food. In the  
215 second set of experiment (DD, referring to “During Digestion”), 1.5 mL of pepsin solution to achieve a  
216 2000 U/mL activity in the mixture (as for the “static” conditions), were added before starting the  
217 experiment. At the end of these experiments, 36.7  $\mu$ L of a 10 mM pepstatin solution (6.859 g/L) were  
218 added in order to ensure a total pepsin inhibition (Umezawa, 1976), a 200  $\mu$ L sample was collected for  
219 further analysis of the degree of hydrolysis using the OPA method, and the pH was risen back to 6.3  
220 with a 6 N NaOH solution ( $\sim$  450  $\mu$ L). The pH-stat experiment was launched once again thereafter to  
221 determine the buffering capacity of the digested test food, hence constituting the third set of experiments  
222 (AD, referring to “After Digestion”). At the end of these last experiments, a 200  $\mu$ L sample was also  
223 collected for further analysis of the degree of hydrolysis using the OPA method.

224

### 225 3.4 Buffering capacity

226 The buffering capacity expresses the resistance of the studied medium to a pH change, and rises  
227 from the sum of all individual acid-base groups. The buffering capacity of the undigested and digested  
228 model foods were calculated from the data collected during the dynamic pH digestions performed before  
229 (BD) and after (AD) pepsin digestion. The calculation was performed at each pH according to the  
230 relation (Salaün, Mietton, & Gaucheron, 2005; Whittier, 1929):

231 *Equation 6*

$$232 \quad BC(pH) = \frac{dB}{dpH} = \frac{(\text{volume of acid added}) \times (\text{normality of the acid})}{(\text{volume of the sample}) \times (\text{pH change produced})}$$

233 with  $BC(pH)$  the buffering capacity at a given pH,  $dB$  the increment of acid added, and  $dpH$  the  
234 associated pH shift.

235

### 236 **3.5 Treatment of data obtained in dynamic pH conditions**

237 All calculations were made using Matlab® software (The MathWorks Inc., Natick, USA). The  
238 main steps of the data treatment procedure were as follows. The times, volumes and pH values recorded  
239 by the pH-stat device in dynamic pH conditions varied from one experiment to another. Recorded pH  
240 values were smoothed with a moving average on 60 points, which corresponds to a 1 min interval (data  
241 acquisition frequency of 1 s<sup>-1</sup>). Volumes and pH values were then linearly interpolated every minute  
242 from 0 to 120 min to enable the calculation of means and standard deviations as a function of time over  
243 the 3 replicates for each set of experiments. To calculate the buffering capacities of undigested and  
244 digested model foods, first order derivative values of the volumes and pH recorded the BD and AD  
245 experiments were calculated for each replicate using the Savitsky-Golay algorithm with a linear fit over  
246 9 points (Savitzky & Golay, 1964). These data were used to solve Equation 6, before calculating means  
247 and standard deviations. Finally, all times, volumes, and buffering capacity values were linearly  
248 interpolated to the mean pH values recorded during the DD experiments. This last step was performed  
249 to enable converting titrant volumes into degree of hydrolysis with Equation 5, and plotting the results  
250 as a function of pH when needed.

251

### 252 **3.6 Degree of hydrolysis measured by the OPA method**

253 Degrees of hydrolysis (DH) of proteins were measured on the samples collected at the end of  
254 all gastric digestion experiments using the OPA (ortho-phthalaldehyde) method, according to the  
255 protocol proposed by (Spellman et al., 2003) with no SDS addition. It consists in measuring the  
256 absorbance at 340 nm of the complex formed between the amino groups of peptides and OPA in the  
257 presence of NAC (N-Acetyl-L-cysteine) with a UV-Vis spectrophotometer (Evolution 201, Thermo  
258 Scientific). The difference of absorbance,  $\Delta Abs$ , between the hydrolyzed and unhydrolyzed samples  
259 therefore provides the number of amino groups produced during the proteolytic reaction (Church, Porter,  
260 Catignani, & Swaisgood, 1985), from which degrees of hydrolysis (%) can be calculated according to:

261 Equation 7

$$262 \quad DH = 100 \times \frac{d \times \Delta Abs}{\varepsilon \times m \times h_{tot}}$$

263 where  $\varepsilon$  is the molar extinction coefficient at 340 nm ( $6363.4 \text{ M}^{-1} \cdot \text{cm}^{-1}$  as obtained from a calibration  
264 curve made with leucine),  $d$  is the dilution factor,  $m$  is the protein mass (g), and  $h_{tot}$  is the quantity of  
265 peptide bonds contained in the studied proteins:  $8.8 \times 10^{-3} \text{ eqv/g}$  for whey proteins (Spellman et al., 2003).

266

## 267 4 Results & Discussion

### 268 4.1 Gastric digestions in static pH conditions (pH = 3)

269 The first aim of this study was to test the efficiency of the pH-stat method to monitor pepsin  
270 activity during a static *in vitro* gastric digestion of a model food containing native whey proteins. Fig. 2  
271 presents the results obtained over 3 repetitions using the protocol of (Minekus et al., 2014). The trend is  
272 typical of an enzymatic reaction, with a high initial rate of reaction followed by a progressive slowdown  
273 (Fig. 2A). During the second hour of digestion, the slow but continuous increase shows that pepsin  
274 activity was low but not null at the end of the experiments. Switching the time axis to a logarithmic  
275 scale (Fig. 2B) clearly gives the indication that the reaction was not over. Moreover, from ~20 min the  
276 kinetics were a linear function of the logarithm of time ( $R^2 = 0.9998$ ). By the end of digestion, a mean  
277 of  $6.95 \pm 0.34 \text{ mL}$  of titrant solution was added.

278 The degree of hydrolysis (DH) estimated using the OPA method on the end samples was  $3.70$   
279  $\pm 0.89 \%$  (Table 1). This value is consistent with the high resistance of native whey proteins to pepsin  
280 hydrolysis, providing that DH is typically around 10-15 % after pepsin digestion (Griffiths, 2012;  
281 Norton et al., 2014). For comparison on whey proteins, (Kim et al., 2007) found values in the range 4-  
282 10% after 2 h of pepsin digestion, and (Asselin et al., 1989) reported a value as low as 1.7 % after a 4 h  
283 experiment. Indeed,  $\beta$ -lactoglobulin, the major protein of whey, has been reported to be rather resistant  
284 to pepsinolysis thanks to its native conformation, particularly stable at low pH (Astwood, Leach, &  
285 Fuchs, 1996; Fu, Abbott, & Hatzos, 2002; Guo, Fox, Flynn, & Kindstedt, 1995; Kitabatake & Kinekawa,

286 1998). Moreover, reversing Equation 3 with a DH of 3.70 % and a titrant volume of 6.95 mL leads to a  
287 mean degree of dissociation of peptide's C-terminus carboxylic groups,  $\alpha_{COOH}$ , of 0.090, which means  
288 that about 91 % of the pepsin activity would be titrated at pH 3. This first estimation will be further  
289 refined and discussed in the light of the results obtained in dynamic pH conditions.

290

## 291 **4.2 Gastric digestions in dynamic pH conditions (from pH 6.3 down to 2)**

### 292 **4.2.1 Titration curves**

293 Fig. 3 shows the evolution of pH and titrant volume during the 3 sets of experiments performed  
294 in dynamic pH conditions, *i.e.* with a progressive acidification of the medium (BD, DD, and AD). The  
295 rate of HCl addition to reach pH = 2.0 was the same in all experiments (0.031 mL/min), as programmed  
296 in the pH-stat experimental setup (Fig. 3A) to enable a progressive acidification of the medium (Fig.  
297 3B). The last stage of the experiments, *i.e.* after pH 2 was reached, corresponds to a classical pH-stat  
298 experiment. The volumes added, and hence time needed, to reach pH 2 slightly varied between  
299 experiments. In the absence of pepsin, 1.90 mL (61 min) of titrant were needed to counteract the  
300 buffering capacity of the medium. This volume did not increase further during the second stage of the  
301 experiment. This demonstrates that no acid hydrolysis of proteins took place, a finding that was further  
302 confirmed by the OPA results (DH =  $-0.57 \pm 0.64$  %, Table 1).

303 The volume added to reach pH 2 increased to 2.12 mL (67 min) in presence of pepsin, hence  
304 reflecting the consumption of protons at acidic pH by the amino groups of the peptides produced. During  
305 the second stage of these experiments, the slow but progressive increase of HCl volume (up to 2.20 mL  
306 at 120 min, Fig. 3A) illustrates the continuity of the pepsin activity. At the end of the experiment, the  
307 DH value was  $3.60 \pm 0.53$  % according to the OPA method (Table 1).

308 During the last set of experiments, launched after the addition of a pepsin inhibitor (pepstatin)  
309 right after the DD experiments, 2.36 mL (75 min) were needed to reach pH 2. This value is slightly  
310 above the 2.20 mL added at the end of the pepsin digestion experiments because of the greater initial  
311 volume of the medium induced by the addition of titrant in the DD experiments, subsequent pH

312 neutralization, and pepstatin addition. Indeed, the constant volume of titrant obtained in the second stage  
313 of the experiment (2.37 mL at 120 min) shows that the residual activity of pepsin in presence of pepstatin  
314 was negligible. This was also confirmed by the OPA method, which provided a final DH of  $2.87 \pm 0.36$   
315 % that is not statistically different from the  $3.60 \pm 0.53$  % estimated at the end of the DD experiments  
316 according to experimental uncertainties (Table 1).

317 As can be seen from Fig. 3B, the pH measured before (BD) and during (DD) pepsin digestion  
318 are starting to diverge around pH 4, at  $t \sim 25$  min, while being undistinguishable before. This could  
319 signal the beginning of pepsin action. However, this observation could also simply reflect the greater  
320 sensitivity of our titration experiments with decreasing pH, because of the concomitant decrease in the  
321 degree of dissociation of the peptides' C-terminus carboxylic groups. To deepen our analyses of the  
322 results and draw clear conclusions, the acid-base equilibrium of these carboxylic groups was more-  
323 rigorously estimated from the buffering capacity of the undigested and digested foods.

324

#### 325 **4.2.2 Buffering capacities of undigested and digested model foods**

326 Fig. 4A presents the buffering capacity of the undigested (green triangles) and digested (red  
327 circles) model foods, as estimated from the BD and AD experimental data, respectively. The buffering  
328 capacity of the undigested food increases from pH 6 up to a local maximum, at  $\text{pH} \sim 3.5$ . This maximum  
329 is consistent with previous results on proteins and is mainly due to the acidic amino acids, which contain  
330 carboxylic groups in their side chains (Salaün et al., 2005; Whittier, 1929). After pepsin digestion, the  
331 buffering capacity increased in the range 2.7-5.3, with a maximum approximately 23 % higher than  
332 before digestion at pH 3.7. This increase reflects the supplemental buffering effect induced by the neo-  
333 formed carboxylic groups at the peptides' C-terminal end, the contribution of which can be estimated  
334 by subtracting the two curves.

335

#### 336 **4.2.3 Acid-base equilibrium of the peptide's C-terminus carboxylic groups as a function of pH**

337 Fig. 4A illustrates the results of this subtraction (blue open squares), which suggest that peptides

338 cannot be detected by acid titration for  $\text{pH} \gtrsim 5$ , and that all of them are titrated below 2.5. They also  
339 show that the  $\text{pK}_A$  of the C-terminus carboxylic groups are broadly distributed around the median of the  
340 mode at  $\text{pH} 4.0$ , which appears consistent with the estimated  $\text{pK}_A$  of 3.75 for the carboxylic groups of  
341 proteins (Nielsen, 1997).

342 Fig. 4B (squares) shows the cumulative buffering capacity of peptides ( $BC_{\text{pep}}^{\text{cumul}}$ ) as a function  
343 of  $\text{pH}$ , for which the negative values observed at low  $\text{pH}$  were neglected. This variable more clearly  
344 illustrates that all peptides are titrated below  $\text{pH} 2.5$ , and was used to estimate the average degree of  
345 dissociation of peptides' C-terminus carboxylic groups ( $\alpha_{\text{COOH}}$ , squares in Fig. 4B) as a function of  $\text{pH}$   
346 according to:

347 Equation 8

$$348 \quad \alpha_{\text{COOH}}(\text{pH}) = 1 - \frac{BC_{\text{pep}}^{\text{cumul}}(\text{pH})}{BC_{\text{pep}}^{\text{cumul}}(\text{pH} = 2)}$$

349

#### 350 4.2.4 Evolution of DH during both types of *in vitro* gastric digestions

351 From the latter relation, we obtained  $\alpha_{\text{COOH}} = 0.080$  at  $\text{pH} 3.0$ , meaning that about 92 % of the  
352 peptides produced were effectively titrated during gastric digestions in static  $\text{pH}$  conditions. Our  
353 previous estimate of 0.090 was thus already very good. The value of 0.080, deduced from a more  
354 rigorous approach, was therefore used in Equation 3 to determine the evolution of DH during the  $\text{pH}$ -  
355 stat experiments at  $\text{pH} 3$ . As shown in Table 1 and Fig. 2 (right-hand y-axis), this leads to a final DH  
356 value of  $3.66 \pm 0.17$  % in excellent agreement with the  $3.70 \pm 0.89$  measured with the OPA method. In  
357 specific enzyme units, pepsin hydrolyzed  $0.17 \mu\text{mol}$  of peptide bonds per min and mg of enzyme over  
358 the 2 h of reaction, and  $1.47 \mu\text{mol}/\text{min}/\text{mg}$  during the first 5 min.

359 The evolutions of DH during the course of the digestions performed in acidifying conditions are  
360 shown as functions of both time and  $\text{pH}$  in Fig. 5A and 5B, respectively. Two slightly different sets of  
361 DH results are presented for comparison purposes. The first set (squares) was obtained assuming that  
362  $\alpha_{\text{COOH}} = 0$  in Equation 5, *i.e.* that the peptides produced were all titrated at all  $\text{pH}$ . The second set

363 (diamonds) was obtained by accounting for the values of  $\alpha_{COOH}$  shown in Fig. 4B. These latter estimates  
364 of DH thus account for the greater sensitivity of acid titration to peptide detection as pH decreases.

365 It can be inferred from Fig. 5A that no, or very little, hydrolysis took place within the first ~ 20  
366 min, and that pepsin became significantly active afterwards, with a degree of hydrolysis that steadily  
367 increased throughout the course of the acidifying kinetics (from DH = 0 up to 2.19 %), and after pH 2  
368 was reached. The final DH was estimated to be  $3.03 \pm 0.40$  %, which fall in-between the  $3.60 \pm 0.53$  %  
369 and  $2.87 \pm 0.36$  % estimated by the OPA method at the end of the DD and AD experiments, respectively  
370 (Table 1). This final DH value is also rather close to the 3.66 % obtained after 2 h at a constant pH of  
371 3.0, *i.e.* with the conditions recommended in (Minekus et al., 2014). Our results therefore suggest that,  
372 with regards to protein hydrolysis, the endpoint of the gastric phase of the standardized protocol remains  
373 close to what can be obtained with acidifying kinetics. As shown in the insert of Fig. 5A, pepsin activity  
374 was also a linear function of the logarithm of time ( $R^2 = 0.9884$ ) after reaching pH 2.0 in the DD  
375 experiments ( $t > 67$  min). According to the slopes of our fittings (Fig. 2B and Fig 5A), pepsin activity  
376 during the second hour of gastric digestion was about 73 % higher in dynamic pH conditions than in  
377 static ones. This finding is rather consistent with the previously-reported 40 % higher pepsin activity at  
378 pH 2.0 than at pH 3.0 with serum albumin as a substrate (Piper & Fenton, 1965). Overall, the greater  
379 activity of pepsin at pH 2 therefore partly, but not entirely, compensated the late start of pepsin  
380 hydrolysis in dynamic pH conditions.

381 When plotted against pH (Fig. 5B), it clearly appears that pepsin started to be active in the pH  
382 range 5-4.5. This is also in very good agreement with the pH dependence of pepsin activity that is  
383 reported in the literature, with almost no activity at pH 5.5 that suddenly rises up to reach 70 % of its  
384 optimal activity at pH 4.5 (Piper & Fenton, 1965). It can also be noted that very little difference was  
385 observed between the approximated (squares) and more rigorous calculus (diamonds) of DH, which  
386 takes into account the evolution *versus* pH of the mean degree of dissociation of the peptides' C-  
387 terminus carboxylic groups. This results from the balance between pepsin activity and the sensitivity of  
388 the method as a function of pH. At high pH, peptide detection by titration is not possible because all  
389 neo-formed carboxylic groups remain protonated. However, since pepsin becomes active at relatively

390 low pH only, a reasonable number of peptides can be detected by titration when they start being  
391 produced (*e.g.*  $\alpha_{COOH} \sim 0.5$  at pH 4, meaning that 50 % of the peptides produced can be detected). The  
392 last term of Equation 5, which accounts for this correction, thus rapidly tends to be negligible. This is  
393 an interesting finding from a practical point of view, because it means that a very good approximation  
394 of the DH evolution can be obtained without considering this variable when willing to undertake *in vitro*  
395 gastric digestion with acidifying kinetics, as was the case in the present study. It only requires running  
396 two experiments (in presence and absence of pepsin) and uses Equation 5 with  $\alpha_{COOH} = 0$ .

397         As a final remark, it is noteworthy that gastric emptying was not undertaken in the latter  
398 experiments, meaning that there was no loss of buffering capacity induced by the removal of chyme  
399 from the gastric compartment. This contrasts with the *in vivo* reality and with what is classically  
400 performed using *in vitro* dynamic digestion systems. These controlled conditions were suitable to  
401 properly estimate the sensitivity of the method as a function of pH, and demonstrate that pepsin activity  
402 can still be assessed during a progressive acidification. Further studies could be undertaken to determine  
403 whether the presented strategy remains sensitive enough and reliable when a concomitant gastric  
404 emptying is performed.

## 405 **5 Conclusions**

406         This study shows that pH-stat titration at low pH is very well adapted to the determination of  
407 the kinetics of protein hydrolysis by pepsin, and probably other aspartic proteases. This approach was  
408 successfully adapted to the continuous monitoring of protein hydrolysis during *in vitro* gastric digestions  
409 of a model of complex food in both static and dynamic pH conditions. Volumes of titrant can be easily  
410 converted into degrees of hydrolysis (DH) since we estimated that 1 mol of added protons directly  
411 corresponds to 1 mol of hydrolyzed peptide bonds for  $\text{pH} \leq 2.5$ , and to about 0.92 mol at  $\text{pH} = 3.0$  for  
412 whey proteins in our experimental conditions. Given the simplicity and the high repeatability of pH-stat  
413 experiments, the proposed experimental strategies might be valuable in gastric *in vitro* digestion studies.

414

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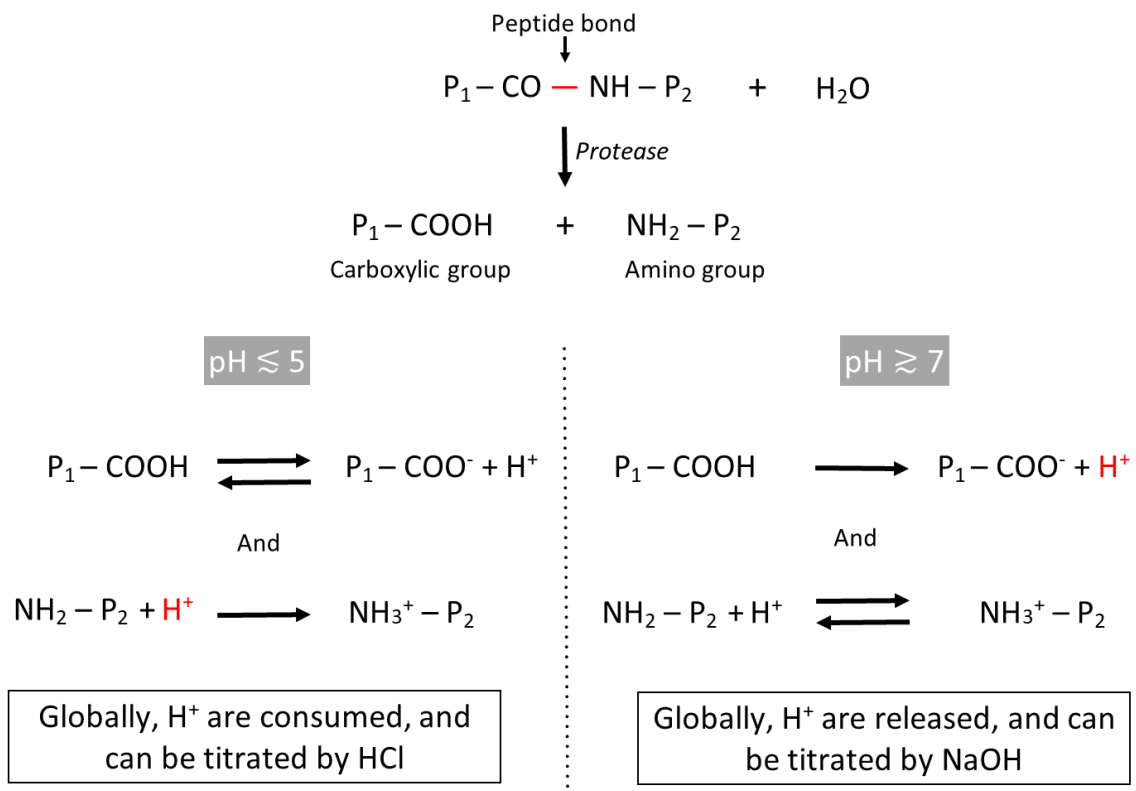
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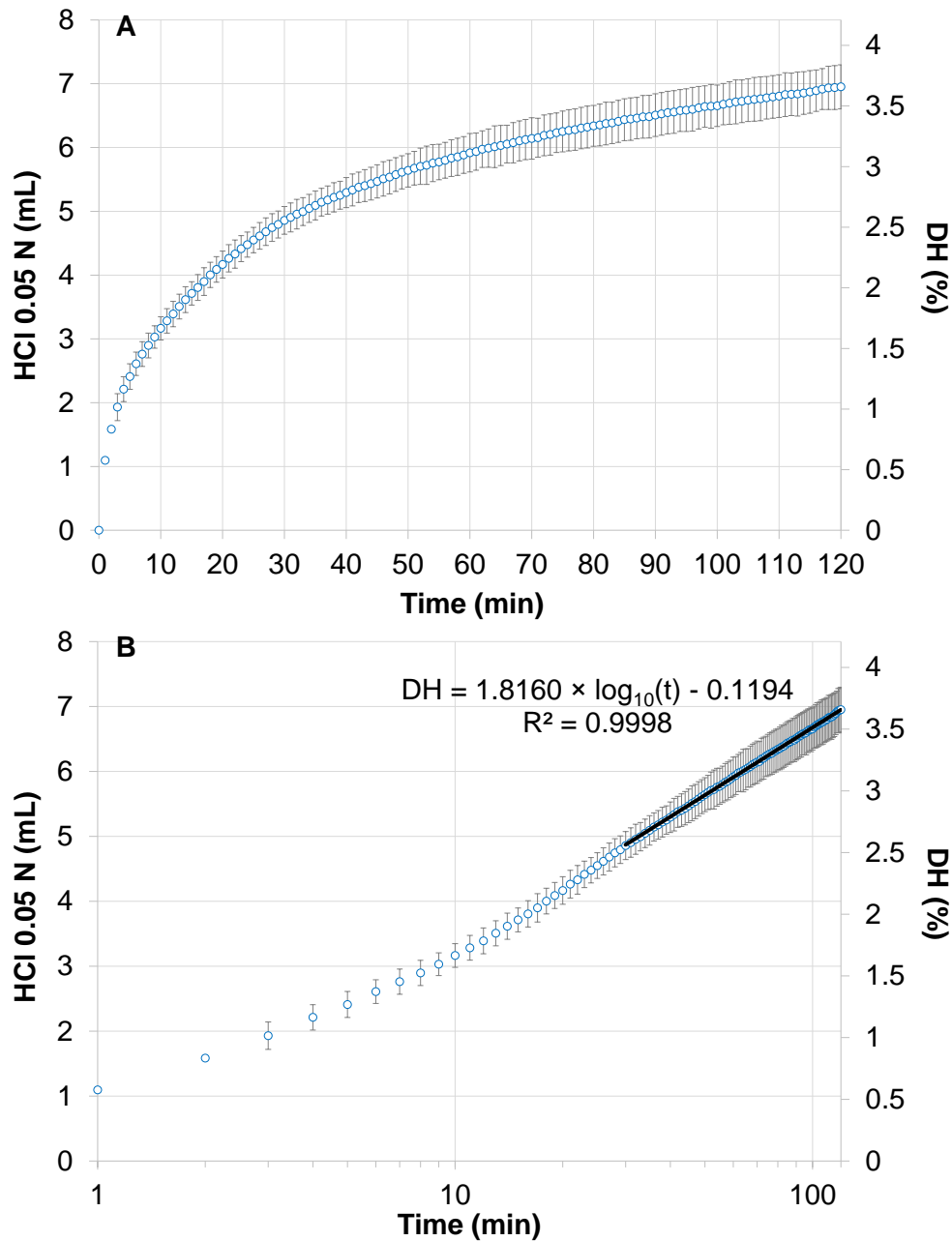
501 **Table 1:** Degree of hydrolysis (%) measured using the OPA method, and pH-stat measurements, at the  
502 end of the different sets of *in vitro* gastric digestions: pH-stat at pH 3, and pH-stat with an acidifying  
503 kinetics before (BD), during (DD), and after (AD) digestion.  
504

	<b>OPA</b>	<b>pH-stat</b>
<b>pH-stat at pH 3.0</b>	$3.70 \pm 0.89$	$3.66 \pm 0.17$
<b>BD experiments</b>	$-0.57 \pm 0.64$	
<b>DD experiments</b>	$3.60 \pm 0.53$	$3.03 \pm 0.40$
<b>AD experiments</b>	$2.87 \pm 0.36$	



505

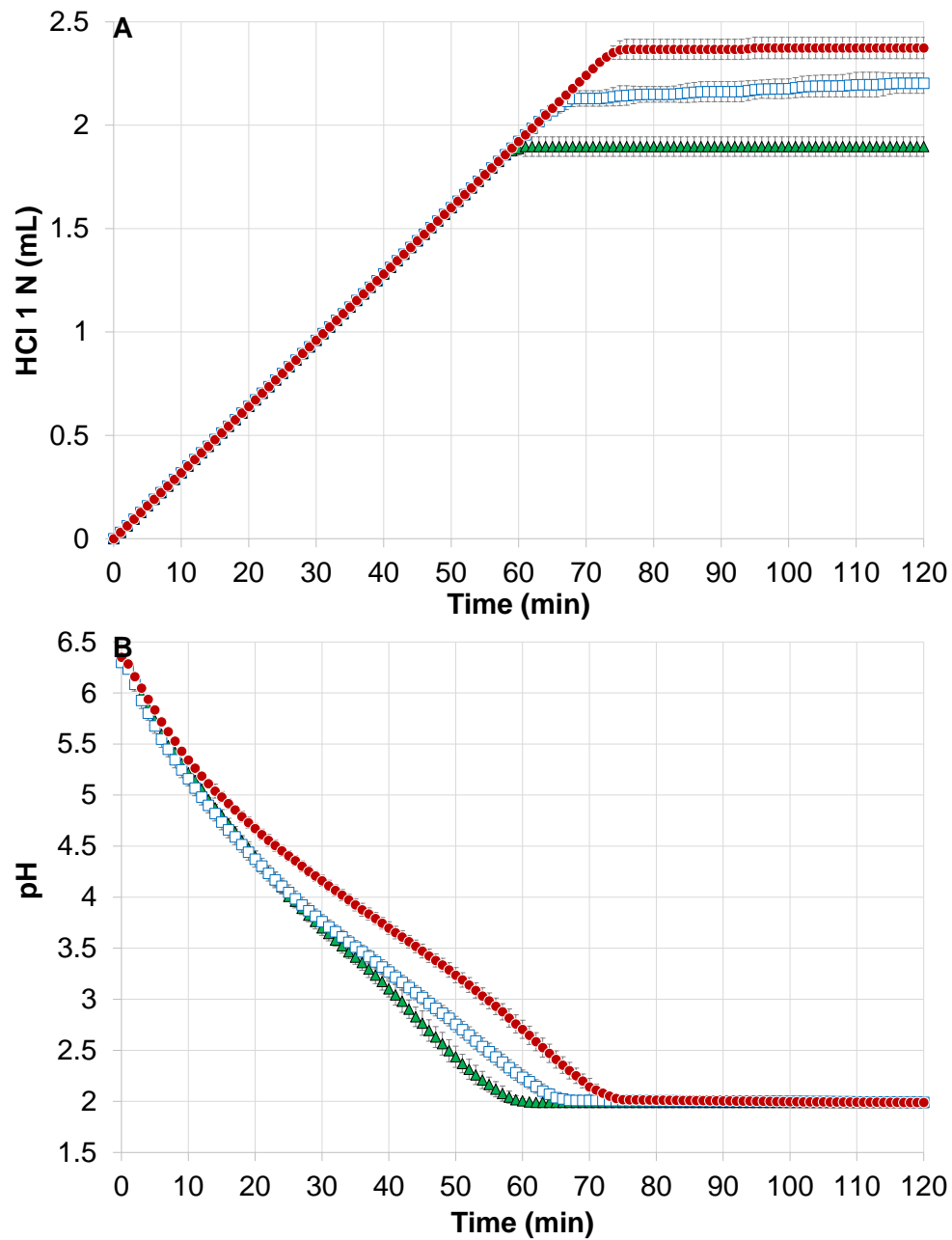
506 **Figure 1:** Schematic representation of protein hydrolysis by a protease, and of the principles of pH-stat  
 507 titration at pH ≳ 7 and pH ≲ 5.



508

509 **Figure 2:** pH-stat acid titration profile of the model food during *in vitro* gastric digestions in static  
 510 conditions (pH = 3): (A) on a linear scale, (B) on a semi-log scale. DH values, on the right-hand y-axes,  
 511 were estimated using  $\alpha_{COOH} = 0.080$  (see section 4.2.3) in Equation 3. Values are means  $\pm$  SD over 3  
 512 repetitions. The black line in Fig. 2B illustrates the fitting result ( $DH = 1.8160 \times \log_{10}(t) - 0.1194$ ,  $R^2$   
 513  $= 0.9998$ ).

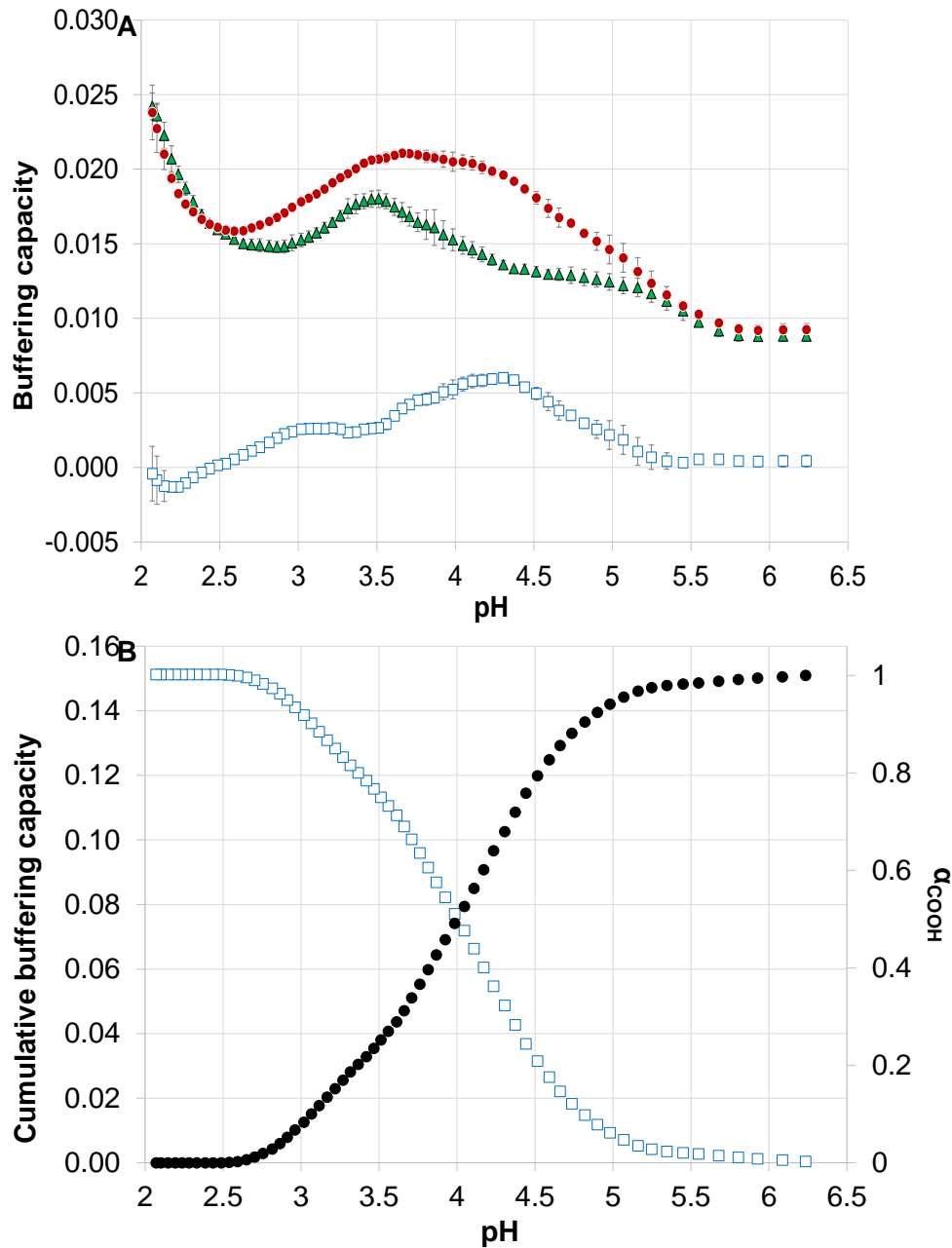
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515

516 **Figure 3:** Evolution of titrant volume (A) and pH (B) during *in vitro* gastric digestions in dynamic pH  
 517 conditions (from pH 6.3 down to 2): before digestion (BD, green filled triangles), during digestion (DD,  
 518 blue open squares), and after digestion (AD, red filled circles). Values are means  $\pm$  SD over 3 repetitions.

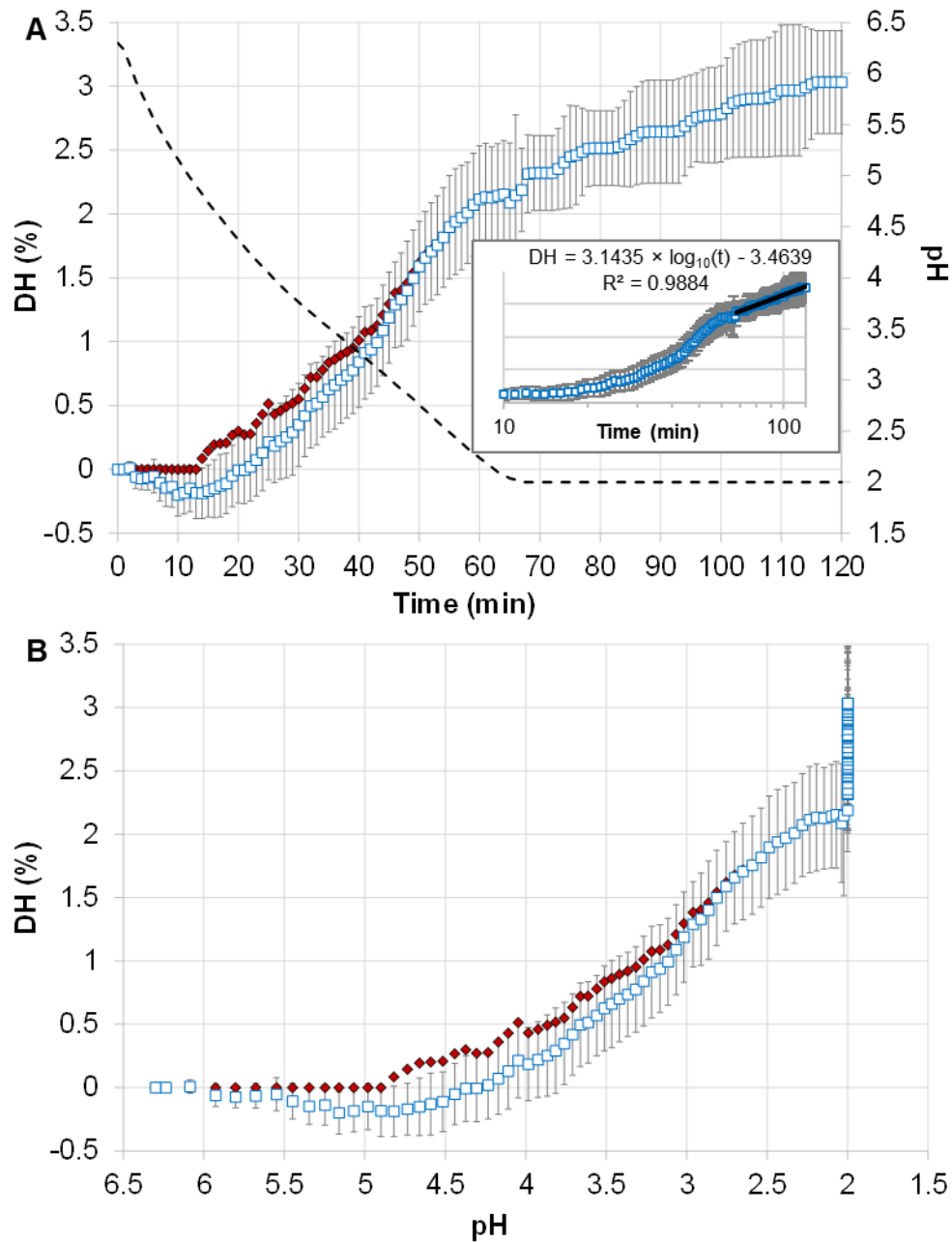
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520

521 **Figure 4:** (A) Buffering capacities of the model food before digestion (BD, green filled triangles), after  
 522 digestion (AD, red filled circles), and their difference (blue open squares) which is indicative of the  
 523 buffering capacity of the neo-formed peptides' C-terminus carboxylic groups. Values are means  $\pm$  SD  
 524 over 3 repetitions. (B) Cumulative buffering capacities induced by the neo-formed peptides (blue open  
 525 squares) and corresponding degree of dissociation of the peptides' C-terminus carboxylic groups ( $\alpha_{COOH}$ )  
 526 according to Equation 8 (black filled circles).

527



528

529 **Figure 5:** Evolution of the degree of hydrolysis (DH) during *in vitro* gastric digestions of the model  
 530 food in dynamic pH conditions as a function of time (Fig. 5A) and pH (Fig. 5B). DH values estimated  
 531 with (red filled diamonds) and without (blue open squares) consideration of  $\alpha_{COOH}$  evolution as a  
 532 function of pH (Equation 5), are both presented. Values are means  $\pm$  SD over 3 repetitions. In Fig. 5A,  
 533 the dashed line shows the pH evolution as a function of time, and the insert shows the evolution of DH  
 534 on a semi-log scale and the fitting result ( $DH = 3.1435 \times \log_{10}(t) - 3.4639$ ,  $R^2 = 0.9884$ ).